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TITLE: Adenoviral vectors for treatment of hemophilia

## BSPR:

Scharfmann et al., Proc. Nat. Acad. Sci., Vol. 88, pgs. 4626-4630 (June 1991) discloses the transduction of mouse fibroblast implants with a retroviral vector including a B-galactosidase gene under the control of the dihydrofolate reductase (DHFR) promoter. Such fibroblasts then were grafted into mice, and expression of the .beta.-galactosidase gene for up to sixty days was obtained. Scharfmann et al. also disclose fibroblasts transduced with canine Factor IX, but they only obtained short-term and non-therapeutic levels of expression.

## BSPR:

Dai et al., Proc. Nat. Acad. Sci., Vol. 89, pgs. 10892-10895 (November 1992) discloses the transfection of mouse primary myoblasts with retroviral vectors including canine Factor IX DNA under the control of a mouse muscle creatine kinase enhancer and a human cytomegalovirus promoter. The transfected myoblasts then were injected into the hind legs of mice. Expression of canine Factor IX over a period of 6 months was obtained; however, the steady-state levels of Factor IX secreted into the plasma (10 ng/ml for 10.sup.7 injected cells) are not sufficient to be of therapeutic value.

## DEPR:

The DNA sequence encoding a clotting factor is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus promoter; the Rous Sarcoma Virus (RSV) promoter; the albumin promoter; inducible promoters, such as the Mouse Mammary Tumor Virus (MMTV) promoter; the metallothionein promoter; heat shock promoters; the .alpha.-1-antitrypsin promoter; the hepatitis B surface antigen promoter; the transferrin promoter; the apolipoprotein A-1 promoter; the Factor VIII promoter; and the Factor IX promoter. It is to be understood, however, that the scope of the present invention is not to be limited to specific promoters.

## DEPR:

In one embodiment, when the DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof, the promoter controlling the DNA sequence is preferably a tissue-specific promoter, such as, for example, the mouse albumin promoter, which is active in liver cells. Although the scope of this embodiment is not intended to be limited to any theoretical reasoning, the inventors believe that, by employing a tissue-specific promoter, possible Factor VIII toxicity to the producer cells is avoided.

## DEPR:

When one employs a mouse albumin promoter, which is active in liver cells, the adenoviral vectors are preferably grown in cells other than liver cells. When the generated adenoviral vectors are to be administered to a host, such vectors are administered to a host by means known to those skilled in the art, whereby the vectors will travel to and infect liver cells. The infected liver cells then will express Factor VIII in therapeutic amounts. Factor VIII is not toxic to liver cells and thus will continue to be expressed at therapeutic levels.

## DEPR:

In yet another embodiment, when the DNA sequence encodes Factor IX or a fragment,

derivative, or analogue thereof, the promoter controlling the DNA sequence is preferably a strong promoter that is not tissue-specific, such as, for example, the Rous Sarcoma Virus promoter. Because it is believed that Factor IX is not toxic to most cells, the adenoviral vectors may be grown in any cell type, and may be administered to a patient in an effective therapeutic amount, whereby the adenoviral vectors will travel to and infect cells such as liver cells, for example, whereby the Factor IX will be expressed in therapeutic amounts.

## DEPR:

In another embodiment, the first exon and first intron of the apolipoprotein A-1 gene may be employed, if desired, with the apolipoprotein A-1 gene promoter. (PNAS, Vol. 80, pgs. 6147-6151 (October 1983); J. Biol. Chem., Vol. 266, No. 27, pgs. 18045-18050 (September 1991)). The above-mentioned introns and/or exons also may be used in combination with the 5' untranslated region and/or the 3' untranslated region of the gene encoding the clotting factor.

## DEPR:

In yet another embodiment, the above-mentioned introns and/or exons and/or promoter of the apolipoprotein A-1 gene may be used in combination with the apolipoprotein A-1 5' untranslated region and/or the apolipoprotein A-1 3' untranslated region and poly A signal. In one embodiment, the above-mentioned introns and/or exons and/or promoter of the apolipoprotein A-1 gene are used in combination with the apolipoprotein A-1 3' untranslated region and poly A signal.

## DEPR:

In a further embodiment, when the DNA sequence encodes Factor VIII or a fragment, derivative, or analogue thereof, the above-mentioned introns and/or exons, and/or promoter of the apolipoprotein A-1 gene may be used in combination with the 5' untranslated region and/or 3' untranslated region and poly A signal of the human Factor IX gene. In one embodiment, the above-mentioned introns and/or exons and/or promoter of the apolipoprotein A-1 gene are used in combination with the 3' untranslated region and poly A signal of the human Factor IX gene.

## DEPR:

In one preferred embodiment, the apolipoprotein A-1 promoter may be employed, alone or in combination with the first exon and/or first intron of the apolipoprotein A-1 gene, in combination with the Factor VIII gene.

## DEPR:

In the preferred embodiment, the adenoviral vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a clotting factor; and a promoter controlling the at least one DNA sequence encoding a clotting factor. The vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, but is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

## DEPR:

Such a vector, in a preferred embodiment, is constructed first by constructing, according to standard techniques, a shuttle plasmid which contains, beginning at the 5' end, the "critical left end elements," which include an adenoviral 5' ITR, an adenoviral encapsidation signal, and an E1a enhancer sequence; a promoter (which may be an adenoviral promoter or a foreign promoter); a multiple cloning site (which may be as hereinabove described); a poly A signal; and a DNA segment which corresponds to a segment of the adenoviral genome. The vector also may contain a tripartite leader sequence. The DNA segment corresponding to the adenoviral genome serves as a substrate for homologous recombination with a modified or mutated adenovirus, and such sequence may encompass, for example, a segment of the adenovirus 5 genome no longer than from base 3329 to base 6246 of the genome. The plasmid may also include a selectable marker and an origin of replication. The origin of replication may be a bacterial origin of replication. Representative examples of such shuttle plasmids include pAVS6, shown in FIG. 5. A desired DNA sequence encoding a clotting factor may then be inserted into the multiple cloning site to produce a plasmid vector.

## DEPR:

Through such homologous recombination, a vector is formed which includes an adenoviral 5' ITR, an adenoviral encapsidation signal; an E1a enhancer sequence;

a promoter; at least one DNA sequence encoding a clotting factor; a poly A signal; adenoviral DNA free of at least the majority of the E1 and E3 adenoviral DNA sequences; and an adenoviral 3' ITR. The vector also may include a tripartite leader sequence. In one embodiment, the tripartite leader sequence is deleted from the adenoviral vector, or the tripartite leader sequence contains one or more mutations such that a polypeptide encoded by such tripartite leader sequence is not expressed. Applicants have found that, by deleting or mutating the tripartite leader sequence of the adenoviral vector, one may obtain improved expression of the clotting factor. The vector may then be transfected into a helper cell line, such as the 293 helper cell line (ATCC No. CRL1573), which will include the E1a and E1b DNA sequences, which are necessary for viral replication, and to generate infectious adenoviral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes.

## DEPR:

Promoters which control the DNA sequence may be selected from those hereinabove described.

## DEPR:

pG1 (FIG. 1), which is a retroviral plasmid vector including a 5' LTR derived from Moloney Sarcoma Virus, a multiple cloning site, and a 3' LTR from Moloney Murine Leukemia Virus, and is described in PCT Application No. W091/10728, published Jul. 25, 1991, was cut with BamHI and HindIII. pLIXSN (Palmer et al, Blood, Vol. 73, No. 2, pgs. 438-445 (February 1989)), which contains a Factor IX gene, an SV40 promoter, and a neo.sup.R gene, was also cut with BamHI and HindIII. The resulting BamHI-HindIII fragment, which contains the Factor IX gene, was then ligated to the BamHI-HindIII digested pG1 to form pG1H9. (FIG. 2). The Factor IX gene could also have been obtained according to the procedures disclosed in U.S. Pat. No. 4,994,371.

## DEPR:

Second, the ITR, encapsidation signal, Rous Sarcoma Virus promoter, the adenoviral tripartite leader (TPL) sequence and linking sequences were assembled as a block using PCR amplification. The ITR and encapsidation signal (sequences 1-392 of Ad-dl327 [identical to sequences from Ad5, Genbank accession #M73260] incorporated herein by reference) were amplified (amplification 1) together from Ad-dl327 using primers containing NotI or AscI restriction sites. The Rous Sarcoma Virus LTR promoter was amplified (amplification 2) from the plasmid pRC/RSV (sequences 209 to 605; Invitrogen, San Diego, Calif.) using primers containing an AscI site and an SfiI site. DNA products from amplifications 1 and 2 were joined using the "overlap" PCR method (amplification 3) (Horton et al., BioTechniques, 8:528-535 (1990)) with only the NotI primer and the SfiI primer. Complementarity between the AscI containing end of each initial DNA amplification product from reactions 1 and 2 allowed joining of these two pieces during amplification. Next the TPL was amplified (amplification 4) (sequences 6049 to 9730 of Ad-dl327 [identical to similar sequences from Ad5, Genbank accession #M73260]) from cDNA made from mRNA isolated from 293 cells (ATCC Accession No. CRL 1573) infected for 16 hrs. with Ad-dl327 using primers containing SfiI and XbaI sites respectively. DNA fragments from amplification reactions 3 and 4 were then joined using PCR (amplification 5) with the NotI and XbaI primers, thus creating the complete gene block.

## DEPR:

The fragment encoding Factor IX was isolated by electrophoresis in a 1.0% agarose gel followed by electroelution of the DNA. This fragment was subcloned into pAvS6 which had been linearized with EcoRV and treated with calf intestinal phosphatase. The resulting shuttle plasmid pAvS6H9B (FIG. 7), contains the 5' inverted terminal repeat of adenovirus type 5 (Ad 5), the origin of replication of Ad 5, the Ad 5 encapsidation signal, the E1a enhancer, the RSV promoter, the tripartite leader sequence of Ad 5, Factor IX cDNA, the SV40 early polyadenylation signal, and Ad 5 sequences from nucleotide positions 3329-6246.

## DEPR:

pAVS6H81 (FIG. 13) was constructed from pMT2LA (FIG. 11) and pAVS6. (FIG. 5). pMT2LA (Genetics Institute, Cambridge, Mass.) includes cDNA encoding a derivative of human Factor VIII in which the B domain of Factor VIII is deleted. Such cDNA is further described in Toole et al., Nature, Vol. 312, pgs. 342-349 (November 1984), Vehar et al., Nature, Vol. 312, pgs. 337-342 (November 1984), and Toole et

al., PNAS, Vol. 83, pgs. 5939-5942 (August 1986). The cDNA is controlled by a Rous Sarcoma Virus promoter. The 4.6 kb cDNA (FIG. 12) contains no natural 5' untranslated DNA, and 216 bp of 3' untranslated DNA. The B domain deletion removes nucleotides 2334-4973 of the coding sequence of the full length Factor VIII. The cDNA for B domain deleted Factor VIII could also have been obtained according to the procedures disclosed in U.S. Pat. No. 4,868,112.

DEPR:

A schematic of the construction of pAVALH81 is shown in FIG. 15. The mouse albumin promoter (Zaret et al., Proc. Nat. Acad. Sci. USA, Vol. 85, pgs. 9076-9080 (1988)), containing 3.5 copies of a liver-specific transcription factor binding site (eG binding sites, Liu et al., Mol. Cell. Biol., Vol. 11, pgs. 773-784 (1991) and Di Persio et al., Mol. Cell. Biol., Vol. 11, pgs. 4405-4414 (1991)) was PCR amplified from pAT2-3eG (FIG. 15, provided by Kenneth Zaret, Brown University, Providence, R.I.) using oligo MGM8.293,

DEPR:

The ITR, encapsidation signal (see construction of pAVS6) and the albumin promoter were removed from pAVAL1 by digestion with NotI (the ends were filled in with T4 DNA polymerase) and SalI, and inserted into pGEM(sac) (FIG. 15), cut with SalI and SmaI to generate pGEMalb (FIG. 15) (pGEM(sac) was created by cutting pGEM (FIG. 15, Promega; Madison, Wis.) with SacI, and blunting the ends with T4 DNA polymerase and religation, thereby removing the SacI site.) A 1914 bp fragment, containing the 5' region of the B-domain deleted factor VIII cDNA was isolated from pMT2LA (FIG. 11) by digestion with BamHI (filling in the 5' end with T4 DNA polymerase) and digestion with XhoI, and inserted into pGEMalb digested with HindIII (filled in with T4 DNA polymerase) and SalI, to generate pGEMalbF8B (FIG. 15). pGEMalbF8B was cut with MluI and SpeI, and the resulting 1556 bp fragment was inserted into pAVS6H81 (FIG. 13), cut with MluI and SpeI, to generate the adenovirus shuttle plasmid, pAVALH81 (FIG. 16). At least 50 bp on either side of the MluI site (nt 429) and SpeI site (nt 1985) have been verified by sequencing of AVALH81 viral DNA (see below). The sequence of the Factor VIII B-domain deleted cDNA has been verified by sequencing of bases 1075 to 5732 from the original plasmid, pMT2LA (FIG. 11) obtained from Genetics Institute. It should be noted that this sequence differs from the sequence reported by Genetics Institute by two bases. One base change, nt 1317 of pMT2LA was reported by Genetics Institute to be a T (Toole et al., Nature, Vol. 312, pgs. 342-347 (1984) and by Wood et al., Nature, Vol. 312, pgs. 330-337 (1984) to be an A. In addition, nt 5721 of pMT2LA, reported by Genetics Institute to be a T, was deleted, thus creating a BamHI site within the Factor VIII 3' untranslated region. This mutation does not change the Factor VIII coding region.

DEPR:

A schematic of the construction of pAVAPH81 is shown in FIG. 17. A 1913 bp fragment was isolated from pAVS6H81 (FIG. 13) by digestion with Bam HI, and inserted into pGEM(sac) (FIG. 15) cut with Bam HI, to create pGemF8B2 (FIG. 17). The ApoA1 promoter, first exon (untranslated), first intron, and second exon to the ATG (Genbank #X07496) were PCR amplified using pBGS19-AIgI (FIG. 17) as the template. pBGS19-AIgI (FIG. 17) was constructed in two steps: 1) The 13 kb SalI fragment was removed from Lambda A1 103 (Swanson, et al., Transgenic Research, Vol. 1, pgs. 142-147 (1992), and inserted into pUC19 (FIG. 17, Gibco BRL) to generate pUC19-AIgI (FIG. 17). 2) The 2 kb SmaI fragment was isolated from pUC19-AIgI (FIG. 30) and inserted into pBGS19 (FIG. 17) to generate pBGS19-AIgI (FIG. 17). pBGS19 (ATCC No. 37437) is a kanamycin analog of pUC19. PCR-amplification of pBGS19-AIgI was performed using oligo SSC1.593,

DEPR:

A Factor IX sequence, which includes 9 bp of the Factor IX promoter, the 5' untranslated region, the coding region, and a 162 bp segment of the 3' untranslated region, was excised from pKG5IX-2 (obtained from George Brownlee, University of Oxford, Oxford England) as a Bam HI to HindIII fragment. This fragment is described further in Anson et al., Nature, Vol. 315, pgs. 683-685 (1985). This insert was inserted into the polylinker of pBluescript II SK+ (Stratagene) to form BLSKH9CI. (FIG. 31). The Factor IX sequences were sequenced completely and verified to be correct. Factor IX DNA with genomic elements could also have been obtained according to the procedures disclosed in U.S. Pat. No. 4,994,371 and European Patent EP 0 107 278 B1.

DEPR:

A fragment containing the downstream part of the coding sequence, the full 3' untranslated region, the native Factor IX polyadenylation signal, and 331 bp past the polyadenylation site was excised from pCMVIXa (provided by Jerry Hsueh, Fudan University, Shanghai, China) with PpuMI and BglIII. The BglIII single strand overhang was blunted. pBLSKH9CI was cut with PpuMI and HindIII, the HindIII site was blunted, and the backbone fragment was joined to the fragment obtained from pCMVIXa as a PpuMI-blunt ligation. The resulting plasmid, pBLSKH9D (FIG. 32), contains the 9 bp of promoter, 5' untranslated region, the entire Factor IX coding sequence, the full 3' untranslated region, natural polyadenylation signal, and 331 bp downstream from the polyadenylation signal.

**DEPR:**

The Factor IX sequences were then excised from pBLSKH9D, pBLH9E, and pBLH9F and inserted into the pAvS6 backbone as SpeI-ClaI fragments. The resulting plasmids were termed pAV1H9D (FIG. 37), pAV1H9E, and pAV1H9F, respectively. However, when pAV1H9E and pAV1H9F were sequenced, errors were found in the 5' untranslated region of the Factor IX gene. These errors were repaired. The sequence errors were traced back to pBLH9EINT. Miniprep one this plasmid had been used to generate the subsequent plasmids. pBLH9EINT miniprep six was found to have the correct sequence. The SpeI to AatII fragment in pBLH9EINT miniprep six was used to replace the corresponding fragment in pAV1H9E and pAV1H9F to yield pAV1H9ER (FIG. 38) and pAV1H9FR (FIG. 39), respectively. These plasmids contain the adenovirus type 5 ITR, RSV promoter, tripartite leader, Factor IX sequence, SV40 polyadenylation site (which is superfluous in pAV1H9D and pAV1H9FR), and adenovirus homologous recombination region.

**DEPR:**

RNA was isolated from mouse livers using the RNazole B (TelTest, Friendswood, Tex.) extraction method. RNase protection analyses were performed using the RNase Protection Kit II (Ambion, Austin, Tex.). For each sample, quantities of 5 to 150  $\mu$ g of total cellular RNA was hybridized for 12 hrs at 45.degree. C. with 5.times.10<sup>4</sup> cpm of a gel-purified RNA probe (see below), digested with the RNase A/T1 solution provided with the kit, diluted 1:100, processed as directed, and analyzed on an 8% polyacrylamide-8M urea gel (SequaGel, National Diagnostics, Atlanta, Ga.). The band intensities were quantitated with a Molecular Dynamics PhosphorImager SF. The values obtained were normalized for the number of G-residues in the protected mRNA fragment, as the antisense RNA probes were synthesized with  $\alpha$ -<sup>32</sup>P-CTP. RNA molecular weight markers were synthesized using the RNA Century Marker Template Set (Ambion, Austin, Tex.). <sup>32</sup>P-labeled fragments from Hpa II digested pBR322 were used as DNA size markers. The FVIII probe template (FIGS. 46 and 48), pGemSRpr, was constructed by inserting the 204 bp Sac I-Eco RI fragment isolated from pMT2LA (provided by Genetics Institute, Cambridge, Mass.) (Toole et al., Prot. Nat. Acad. Sci., Vol. 83, pg. 5939 (1986) into pGem4Z (Promega, Madison, Wis.) cut with Sac I and Eco RI. The ALAPH81 probe template (FIG. 47), pGEMALAPF8pr, was created by digesting pAVALAPH81 (FIG. 20) with Mse I (filled in with T4 DNA polymerase) and Eco RI. This 506 bp fragment containing part of the albumin promoter, the ApoA1 first exon, first intron, second exon, and FVIII coding region sequences was inserted into pGem4Z (Promega, Madison, Wis.) digested with Sma I and Eco RI. The ALH81 probe template (FIG. 47), pGemF8probe, has been described (Connelly et al., 1995). The mouse GAPDH-specific and mouse actin-specific probe templates were generated from the pTRI-GAPDH mouse plasmid (Ambion, Austin, Tex.) and pTRI-ACTIN mouse plasmid (Ambion, Austin, Tex.) digested with Sty I and Hind III, respectively. The FVIII-specific probe templates were linearized with Hind III and all anti-sense RNA probes were synthesized with SP6 polymerase and  $\alpha$ -<sup>32</sup>P-CTP (Amersham, Arlington Heights, Ill., 3000 Ci/mmol).

**DEPR:**

To verify that transcription from the albumin promoter initiated at the predicted site in vivo (Gorski et al., Cell, Vol. 47, pg. 767, 1986; Connelly et al., 1995), an RNase protection analysis was performed using an anti-sense RNA probe containing sequences from the albumin promoter and the 5' end of the BDD FVIII cDNA (FIG. 47B; Connelly et al., 1995).

**DEPR:**

FIG. 47A depicts the RNase protection analysis results. RNA samples isolated from mice that received Av1ALH81 (Lanes 4-7) were analyzed using the ALH81 probe. The arrow labeled initiation designates the 247 nt protected probe fragment indicating transcripts properly initiated at the albumin promoter. RNA isolated

from Av1ALAPH81-injected mice (Lanes 11-14) was analyzed using the ALAPH81 probe. The 471 nt and 239 nt protected probe fragments, representing unspliced and spliced transcripts, respectively, are indicated. Liver RNA purified from a mouse injected with the control vector, Av1ALH9B, analyzed with ALH81 (Lane 3) and ALAPH81 (Lane 10) probes served as the negative control. Lane 1 contains .sup.32 P-labeled RNA molecular weight markers. Lanes 8 and 15 contain .sup.32 P-labeled DNA molecular weight markers. Lanes 3 and 9 contain undigested, full-length ALH81 and ALAPH81 probes, respectively. FIG. 47B is a schematic diagram depicting the probe templates and complementary mRNA fragments. The boxes marked Alb and SP6 represent the albumin and SP6 promoter regions, respectively. The rightward pointing arrow indicates the site of transcription initiation from the albumin promoter. The leftward pointing arrow indicates the direction of transcription from the SP6 promoter. The solid black boxes represent the Apo A1 genomic sequences.

DEPR:

Analysis of liver RNA samples isolated from mice injected with Av1ALH81 revealed a fragment of the predicted size for transcripts properly initiated at the albumin promoter (247 nts; FIG. 47B, lanes 4-7; Connelly et al., 1995), and was not found in RNA isolated from Av1ALH9B-transduced mouse liver (FIG. 47A, lane 3). The accuracy and efficiency of splicing of Av1ALAPH81-derived transcripts were evaluated using an anti-sense RNA probe capable of distinguishing unspliced FVIII mRNA from spliced mRNA (FIG. 47B), in an RNase protection analysis (FIG. 47A). With this probe, unspliced RNA would protect a fragment of 471 nts. Transcripts accurately spliced would produce two protected fragments, one 34 nts, representing the ApoA5' exon, and a fragment of 239 nts derived from the 3' exon and FVIII coding region. All Av1ALAPH81-derived RNA samples contained protected fragments representing unspliced and spliced transcripts in approximately equal amounts (FIG. 47A, lanes 11-14). Quantitation by phosphorimager scanning, and adjustment of the values for the number of G residues in each protected fragment showed that an average of 69% of the transcripts were spliced. Therefore, transcription from the albumin promoter initiated at the predicted site in vivo, and accurate splicing of the Apo A1 intron sequences from the FVIII pre-mRNA occurred in the mouse livers.

DEPR:

Liver-specific Expression of the Albumin Promoter

DEPR:

The modified mouse albumin promoter (Hafenrichter et al., Blood, Vol. 10, pg. 3394 (1994); Connelly et al., 1995) had been shown to direct a high level of liver-specific expression in a conditionally transformed hepatocyte cell line, H2.35 (Zaret et al., 1998), under differentiating conditions (DiPersio et al., 1991), and was active when transferred to rat hepatocytes in vivo, in the context of a retroviral vector (Hafenrichter et al., 1994). However, the function of the mouse albumin promoter in vivo, when incorporated into an adenoviral vector backbone had not been determined. To ascertain whether expression from the albumin promoter was tissue-specific, an RNase protection analysis was performed using RNA isolated from several Av1ALAPH81-transduced mouse organs. It had been shown previously, however, that intravenous injection of adenoviral vectors to mice resulted in preferential accumulation of vector in the liver with other organs having lower transduction efficiencies (Smith et al., Nature Genetics, Vol. 5, pg. 397 (1993)). Therefore, first it was necessary to determine the relative transduction efficiencies of the different organs, and then normalize the RNA concentrations for the RNase protection assay dependent upon the organ transduction efficiency. DNA was isolated from liver, lung, and spleen derived from the Av1ALAPH81-injected mice one week post injection, and the vector copy number per cell was assessed by Southern analysis (FIG. 48A).

DEPR:

Using an anti-sense RNA probe complimentary to the FVIII coding region, a FVIII-specific protected fragment of the predicted size (212 nts; FIG. 48C) was detected only in the liver RNA samples (FIG. 48B, lanes 4-7). No FVIII-specific RNA was found in the lung or spleen RNA samples (FIG. 48B, lanes 9-12, and lanes 14-17), or in the Av1ALH9B-transduced control liver RNA (FIG. 48B, lane 3). Analysis of equal quantities of RNA (10 .mu.g) from all samples with an anti-sense RNA probe specific to the mouse actin coding region verified the integrity of the RNA within each of the samples (FIG. 48B, lower panel). Notably, the differences in the actin RNA levels observed in the liver, lung, and spleen

samples probably reflects the normal variation in the endogenous expression levels of actin within the three organs. Therefore, the albumin promoter incorporated into an adenoviral vector directed the expression of BDD FVIII in a liver-specific manner.

DEPR:

The loss in detectable levels of FVIII in the mouse plasma 17 to 22 weeks after vector administration could be caused by several factors. For example, vector DNA may have been eliminated from the liver, the albumin promoter could have become inactivated, or the mice may have developed an antibody response directed against human FVIII. To distinguish between these possibilities, mouse liver DNA and RNA obtained from mice injected with 4.times.10.sup.9 pfu or 5.times.10.sup.8 pfu of Av1ALAPH81 one and 22 weeks after vector administration was analyzed (FIG. 51). Groups of mice that received a dose of 4.times.10.sup.9 pfu or 5.times.10.sup.8 pfu of Av1ALAPH81 were sacrificed at one week or at 22 weeks after vector administration. Liver DNA and RNA were isolated from each mouse liver. FIG. 51A shows Southern analysis of mouse liver DNA isolated from mice that received 4.times.10.sup.9 pfu of Av1ALAPH81. Each DNA sample was digested with BamHI, and subjected to Southern analysis. The arrow designates a 3.4 kb fragment containing Av1ALAPH81-derived Factor VIII sequences. The standards were generated by digesting purified Av1ALAPH81 viral DNA in amounts equivalent to 25, 10, and 1 vector copies per cell. DNA marker sizes are indicated in kb. For mice that received the high vector dose, Southern analysis of liver DNA with comparison to vector copy number standards of 25, 10, and 1 copies (FIG. 51A, lanes 1-3) showed an average of 45 vector copies per cell, at one week post injection (FIG. 51A, lanes 6-9), compared to an average of 0.2 copies per cell at 22 weeks (FIG. 51A, lanes 10-13) revealing that the majority of vector DNA had been lost from the mouse livers. No FVIII-containing vector was detected in an uninjected control mouse liver DNA sample (FIG. 51A, lane 4), or a liver DNA sample isolated from a mouse injected with 4.times.10.sup.9 pfu of Av1ALAPH81 (FIG. 51A, lane 5).

DEPR:

RNAse protection analysis using RNA isolated from the mouse livers (FIG. 51D), and the human FVIII-specific anti-sense RNA probe revealed a high level of FVIII-specific mRNA at both one and 22 weeks after vector administration. 50 .mu.g of total cellular RNA isolated from livers of mice which received 5.times.10.sup.8 pfu of Av1ALAPH81 were used in each reaction. The arrow labeled FVIII designates the 212 nt human Factor VIII specific protected probe fragment. The lanes marked Neg contain RNA isolated from an uninjected control mouse, and a mouse that received a similar dose of Av1ALH9B. The lane marked P contains undigested full-length probe. The lane marked M contains .sup.32 P-labeled DNA molecular weight markers. The lower panel displays a separate RNAse protection assay using 20 .mu.g of total cellular mouse liver RNA, and an antisense RNA probe encoding a portion of the mouse GAPDH cDNA. The arrow labeled GAPDH designates the 134 nt mouse GAPDH-specific protected fragment. Quantitation by phosphorimager scanning showed only a 3-fold decrease in the FVIII mRNA levels from 1 to 22 weeks. No human FVIII-specific protected probe fragment was detected in the uninjected negative control RNA sample (FIG. 51D, lane 2) or with the Av1ALH9B-injected control mouse liver sample (FIG. 51D, lane 3). As an internal RNA standard, the mouse liver RNA samples were analyzed in a separate RNAse protection assay using the mouse GAPDH anti-sense RNA probe (FIG. 51D, lower panel). Since a significant amount of vector DNA remained in the mouse livers, and a high level of FVIII-specific RNA was detected at 22 weeks after vector administration, it is probable that loss of FVIII expression by 22 weeks was not due to a loss of vector DNA from the mouse livers, or to albumin promoter inactivation.

DEPR:

To determine if the decline in FVIII expression levels in the dog's plasma by days 5 to 8 after vector administration was due to loss of vector DNA from the liver or due to transcriptional inactivation of the albumin promoter, an open biopsy was performed 8 days after treatment. DNA and RNA were isolated from liver and spleen samples collected during the biopsy, and used in Southern and RNAse Protection analyses. DNA was isolated from liver and spleen biopsy samples using standard procedures. Briefly, organ sections were minced and incubated overnight in SDS/Proteinase K buffer. This was followed by three phenol/chloroform extractions, one chloroform extraction, ethanol precipitation and resuspension in water. 20 .mu.g of each DNA sample were digested with Bam HI and subjected to Southern analysis, results of which are shown in FIG. 56. The probe, prepared by



random oligonucleotide priming, contained human FVIII cDNA sequences from +73 to +1345 (Toole et al., 1984; Wood et al., 1984). The copy number control standards were prepared by adding 1.2 ng, 120 pg or 12 pg of viral DNA, equivalent to 10, 1, and 0.1 vector copies per cell, respectively, to 20 .mu.g of normal dog control liver genomic DNA and digesting with BamHI. The band intensities were quantitated with a Molecular Dynamics PhosphorImager SF. A high level of vector-specific DNA was detected in the liver and spleen biopsy samples, approximately 5 and 10 vector copies per cell, respectively. No vector DNA was observed in a liver biopsy sample collected from a normal dog, or from a pre-injection liver biopsy sample collected from the dog.

#### DEPR:

To perform the RNA analysis, RNA was isolated from biopsy samples using the RNazole B (Tel-Test, Friendswood, Tex.) extraction method. RNase protection analyses were performed using the RNase Protection Kit II (Ambion, Austin, Tex.). For each sample, quantities of 50 .mu.g of total cellular RNA were hybridized for 12 hrs at 45.degree. C. with 5.times.10.sup.4 cpm of a gel-purified RNA probe (See below.), digested with the RNase A/T1 solution provided with the kit, diluted 1:100, processed as directed, and analyzed on an 8% polyacrylamide-8 M urea gel (SequaGel, National Diagnostics, Atlanta, Ga.). The band intensities were quantitated with a Molecular Dynamics PhosphorImager SF. The FVIII probe template, pGemSRpr, was constructed by inserting the 204 bp Sac I-Eco RI fragment isolated from pMT2LA (provided by Genetics Institute, Cambridge, Mass.) (Toole et al., 1986) into pGem4Z (Promega, Madison, Wis.) cut with Sac I and Eco RI. The RNA analysis revealed the presence of a high level of FVIII-specific RNA only in the liver biopsy sample. (FIG. 56.) No FVIII-specific RNA was detected in the spleen biopsy sample, the preinjection liver sample, or in the normal dog liver RNA sample. To verify the integrity of the RNA in each sample, a separate RNase protection analysis was performed using a mouse-specific glyceraldehyde 3-phosphate dehydrogenase (GAPDH) probe. The GAPDH probe template was generated from the pTRI-GAPDH mouse plasmid (Ambion, Austin, Tex.) digested with Sty I. A similar amount of RNA was detected in each of the liver RNA samples. However, less GAPDH-specific RNA was detected in the spleen-derived RNA sample, probably reflecting the normal variation in GAPDH levels between different organs. These results indicate that the albumin promoter remained transcriptionally active 8 days after vector administration. In addition, consistent with previous observations in mice, these data reveal that the albumin promoter functioned in a liver-specific manner in the dog, as no FVIII-specific RNA was detected in the spleen biopsy sample although the spleen contained more vector copies per cell than the liver. Taken together, these results indicate that the loss of FVIII expression by 8 days after treatment was not due to a complete loss of vector DNA from the treated animal, nor to the transcriptional inactivation of the albumin promoter. Previous studies have shown that FVIII-deficient dogs have a high likelihood of developing human FVIII inhibitory antibodies, (Littlewood and Barrowcliffe, 1987; Thrombosis and Haemostasis, Vol. 57, pages 314-321). Therefore, the drop in FVIII plasma levels between 5 to 8 days after treatment may have been due to the presence of FVIII inhibitory antibodies in the dog's plasma.

#### DEPR:

The adenoviral vector Av1H9FR was reconstructed to remove an open reading frame (orf) in the tripartite leader (TPL) between the RSV promoter and the Factor IX cDNA. FIG. 60 shows a diagram of the left end of Av1H9FR. The vector begins with natural adenovirus serotype 5 (Ad5) sequences, starting with the inverted terminal repeat (ITR) followed by the packaging signal (.psi.) and the E1a enhancer (E1a enh). This is followed by the RSV promoter and the Ad5 TPL. Downstream of the TPL is the human Factor IX cDNA (huFIX). In Av1H9B, Av1H9D, Av1H9ER, and Av1H9FR, an ATG in the context of a reasonably good Kozak consensus sequence is situated immediately upstream of the TPL. A 63 amino acid open reading frame (orf) follows the ATG. Translation initiation at this ATG would likely have a strong deleterious effect on translation of the Factor IX cDNA.

#### DEPR:

The first step in the construction of Av1H9F1 was to delete the TPL from the shuttle plasmid pAv1H9FR (FIG. 39). This was accomplished by digesting the plasmid with the restriction enzymes SfiI and SpeI. The resulting DNA fragments were subjected to electrophoresis in an agarose gel and the larger of the two fragments was recovered by electroelution. The ends of the DNA were made blunt by treatment with T4 DNA Polymerase, then the fragment was circularized by ligation.



An aliquot of the ligation mixture was used to transform competent DH5 E. coli, and ampicillin-resistant colonies were isolated. Several colonies were amplified and miniprep DNA was analyzed by restriction enzyme digestion. A clone with the correct restriction pattern was identified and expanded. The resulting shuttle plasmid, pAvS15H9F, was co-transfected with the large DNA fragment of ClaI digested Ad-dl327 into 293 cells. Two weeks later, infectious recombinant adenoviral vector plaques were picked, expanded, and screened for expression of Factor IX by ELISA. One positive clone was purified by isolating a single plaque, then amplified. The resulting recombinant adenoviral vector was called Av1H9F1. Its integrity was verified by restriction enzyme diagnostics. The structure of the left end of this vector is shown in FIG. 60. The extreme left end of the vector contains the normal sequence of adenovirus serotype 5 (Ad5), including the inverted terminal repeat (ITR). This region is followed by the RSV promoter, which is immediately followed by the human Factor IX cDNA. The ATG shown in the schematic for Av1H9F1 represents the Factor IX start codon.

DEPR:

Subsequently, 293 cells were cotransfected with pAvS17H9F and the large DNA fragment of ClaI digested Ad-dl327. Recombinant adenoviral vector plaques were picked, expanded, and screened for expression of Factor IX by ELISA. A positive clone was identified and amplified, thus generating the vector Av1H9F2. A schematic of the left end of the vector is shown in FIG. 60. Av1H9F2 identical to Av1H9FR, except for a 5 base pair deletion at the beginning of the TPL, which effectively changes the ATG into a CTG. The structure of the vector was verified by restriction enzyme diagnostics and by DNA sequence analysis of the region between the RSV promoter and the 3' untranslated region of the Factor IX cDNA.

DEPL:

complementary to nts 5231-5212 of pAT2-3eG with the addition of BamHI, ClaI, and SalI restriction sites, as the 3' oligo. The PCR product was cut with MluI and BamHI and inserted into pAVS6 (FIG. 5) cut with MluI and BamHI to generate pAVAL1 (FIG. 15). The sequence of the 964 bp PCR-generated albumin promoter has been verified by sequencing. In addition, at least 50 bp on either side of the MluI site (nt 428) and BamHI site (nt 1392) in pAVAL1 (FIG. 15) have also been verified by sequencing. The plasmid pAT-2-3eG is prepared according to the procedures disclosed in DiPersio et al., Mol. Cell. Biol., 11:4405-4414 (1991) and Zaret et al., Proc. Nat. Acad. Sci., Vol. 85, pgs. 9076-9080 (1988), which disclose the preparation of a mouse albumin promoter with two copies of a liver-specific transcription factor binding site. The plasmid pAT2-3eG has been deposited under the Budapest Treaty in the American Type Culture Collection, 1230 Parklawn Drive, Rockville, Md. 20892, and assigned accession number 69603.

DEPL:

complementary to human factor VIII (Genbank #K01740, nts 151-165 (to the SacI site), and nts 463-487 of pBGS19-AIGI, complementary to the ApoA1 gene (Genbank #X07496) with the addition of a SacI and an EcoRI site. The PCR fragment was digested with XbaI and SacI and the resulting 509 bp fragment was inserted into pGemF8B2 (FIG. 17) digested with XbaI-SacI, to generate pGemAPF8B (FIG. 17). pGemAPF8B was then digested with MluI-SpeI, and the resulting 1084 bp fragment was ligated into pAVS6H81 (FIG. 18) cut with MluI and SpeI, to generate the shuttle plasmid, pAvAPH81 (FIG. 18). The sequence of pAvAPH81, from nts 290 to 1619, which include the PCR-generated ApoA1 promoter region, and all cloning junctions, has been verified.

CLPR:

4. The vector of claim 3 wherein said vector further includes a tissue-specific promoter.

CLPR:

5. The vector of claim 4 wherein said tissue-specific promoter is the mouse albumin promoter.

CLPR:

9. The vector of claim 8 wherein said vector includes the ApoA1 promoter.

CLPR:

15. The vector of claim 14 wherein said vector further includes a promoter which is not a tissue-specific promoter.

CLPR:

16. The vector of claim 15 wherein said promoter is a Rous Sarcoma Virus promoter.

CLPR:

27. The vector of claim 1 wherein said vector comprises adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a clotting factor; and a promoter controlling said at least one DNA sequence encoding a clotting factor, wherein said vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, and is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

CLPR:

35. The cell of claim 33 wherein said vector further includes a tissue-specific promoter.

CLPR:

36. The cell of claim 35 wherein said tissue-specific promoter is the mouse albumin promoter.

CLPR:

39. The cell of claim 38 wherein said vector includes the ApoA1 promoter.

CLPR:

43. The cell of claim 41 wherein said vector further includes a promoter which is not a tissue-specific promoter.

CLPR:

44. The cell of claim 43 wherein said promoter is a Rous Sarcoma Virus promoter.

CLPR:

65. The method of claim 54 wherein said vector comprises an adenoviral 5' ITR; an adenoviral 3' ITR; an adenoviral encapsidation signal; at least one DNA sequence encoding a clotting factor; and a promoter controlling said at least one DNA sequence encoding a clotting factor, wherein said vector is free of at least the majority of adenoviral E1 and E3 DNA sequences, and is not free of all of the E2 and E4 DNA sequences, and DNA sequences encoding adenoviral proteins promoted by the adenoviral major late promoter.

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L6: Entry 4 of 31

File: USPT

Apr 17, 2001

DOCUMENT-IDENTIFIER: US 6217860 B1

TITLE: Gene therapy for solid tumors, papillomas and warts

CCXR:

514/44

ORPL:

Zhang, J.F., et al.; Treatment of a human breast cancer xenograft with an adenovirus vector containing an interferon gene results in rapid regression due to viral oncolysis and gene therapy; Proc. Natl. Acad. Sci. USA; 93:4513-4518 (1996):

**WEST**

Generate Collection

L6: Entry 8 of 31

File: USPT

Oct 3, 2000

DOCUMENT-IDENTIFIER: US 6127175 A

TITLE: Cells for the production of recombinant adenoviruses

## BSPR:

As indicated above, adenoviruses constitute vectors for the transfer of genes which are very efficient for gene and cell therapy applications. For that, a heterologous nucleic acid sequence whose transfer and/or expression into a cell, an organ or an organism is desired may be inserted into their genome. This sequence may contain one or more therapeutic genes, such as a gene whose transcription and possible translation in the target cell generate products having a therapeutic effect. Among the therapeutic products, there may be mentioned more particularly enzymes, blood derivatives, hormones, lymphokines: interleukins, interferons, TNF and the like (FR 9203120), growth factors, neurotransmitters or their precursors or synthesis enzymes, trophic factors: BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5 and the like; apolipoproteins: ApoAI, ApoAIV, ApoE and the like (WO94/25073), dystrophin or a minidystrophin (WO93/06223), tumor suppressor genes: p53, Rb, Rpl1A, DCC, k-rev and the like (WO94/24297), genes encoding factors involved in coagulation: factors VII, VIII, IX and the like, suicide genes: thymidine kinase, cytosine deaminase and the like, or alternatively all or part of a natural or artificial immunoglobulin (Fab, ScFv and the like, WO94/29446), and the like. The therapeutic gene may also be an antisense gene or sequence, whose expression in the target cell makes it possible to control the expression of genes or the transcription of cellular mRNAs. Such sequences can for example be transcribed, in the target cell, into RNAs which are complementary to cellular mRNAs and can thus block their translation into protein, according to the technique described in Patent EP 140 308. The therapeutic gene may also be a gene encoding an antigenic peptide, capable of generating an immune response in man, for the production of vaccines. They may be especially antigenic peptides specific for the Epstein-Barr virus, the HIV virus, the hepatitis B virus (EP 185 573), the pseudo-rabies virus, or specific for tumors (EP 259 212).

CCXR:

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Generate Collection

L6: Entry 13 of 31

File: USPT

Sep 14, 1999

DOCUMENT-IDENTIFIER: US 5952221 A

TITLE: Adeno-associated virus vectors comprising a first and second nucleic acid sequence

## ABPL:

The use of recombinant adeno-associated virus (AAV) virions for the treatment of solid tumors is disclosed. The invention provides for the use of recombinant AAV virions to deliver an AAV vector containing a drug-susceptibility gene and a second gene capable of providing an ancillary effect to solid tumor cells. The second gene can be used to enhance the immunogenicity of the transduced tumor cell. Alternatively, the second gene can be used to provide a tumorstatic effect. The invention also provides for the use of recombinant AAV virions to deliver an interferon gene, or a tumor suppressor gene to provide a therapeutic effect in a transduced tumor cell.

## CCXR:

514/44

## ORPL:

Lauret et al., "Development of Methods for Somatic Cell Gene Therapy Directed Against Viral Diseases, Using Retroviral Vectors Carrying the Murine or Human Interferon-.beta. Coding Sequence: Establishment of the Antiviral State in Human Cells," Human Gene Therapy 4:567-577 (1993).

**WEST**

Generate Collection

L6: Entry 14 of 31

File: USPT

Aug 10, 1999

DOCUMENT-IDENTIFIER: US 5935935 A

TITLE: Adenoviral vectors for treatment of hemophilia

## DEPR:

DNA sequences encoding therapeutic agents which may be placed into the adenoviral vector include, but are not limited to, DNA encoding Factor VIII and Factor IX as hereinabove described; DNA encoding cytokines; DNA sequences encoding tumor necrosis factor (TNF) genes, such as TNF-.alpha.; genes encoding interferons such as Interferon-.alpha., Interferon-.beta., and Interferon-.gamma.; genes encoding interleukins such as IL-1, IL-1.beta., and Interleukins 2 through 14; genes encoding GM-CSF; genes encoding adenosine deaminase, or ADA; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; genes encoding soluble CD4; T-cell receptors; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin (.alpha.1AT) gene, the ornithine transcarbamylase (OTC) gene, the CFTR gene, the insulin gene, viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, and antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A non-B virus.

CCOR:

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**WEST****Generate Collection****Search Results - Record(s) 31 through 31 of 31 returned.**☐ 31. Document ID: US 5552309 A

L6: Entry 31 of 31

File: USPT

Sep 3, 1996

DOCUMENT-IDENTIFIER: US 5552309 A

TITLE: Use of polyols for improving the introduction of genetic material into cells

## BSPR:

DNA sequences encoding therapeutic agents may be placed into the adenoviral vector include, but are not limited to, DNA sequences encoding tumor necrosis factor (TNF) genes, such as TNF-.alpha.; genes encoding interferons such as Interferon-.alpha., Interferon-.beta., and Interferon-.gamma.; genes encoding interleukins such as IL-1, IL-1.beta., and Interleukins 2 through 14; genes encoding GM-CSF; genes encoding adenosine deaminase, or ADA; genes which encode cellular growth factors, such as lymphokines, which are growth factors for lymphocytes; genes encoding epidermal growth factor (EGF), and keratinocyte growth factor (KGF); genes encoding soluble CD4; Factor VIII; Factor IX; T-cell receptors; the LDL receptor, ApoE, ApoC, ApoAI and other genes involved in cholesterol transport and metabolism; the alpha-1 antitrypsin (.alpha.1AT) gene, the ornithine transcarbamylase (OTC) gene, the CFTR gene, the insulin gene, viral thymidine kinase genes, such as the Herpes Simplex Virus thymidine kinase gene, the cytomegalovirus virus thymidine kinase gene, and the varicella-zoster virus thymidine kinase gene; Fc receptors for antigen-binding domains of antibodies, antisense sequences which inhibit viral replication, such as antisense sequences which inhibit replication of hepatitis B or hepatitis non-A non-B virus; antisense c-myc oligonucleotides; and antioxidants such as, but not limited to, manganese superoxide dismutase (Mn-SOD), catalase, copper-zinc-superoxide dismutase (CuZn-SOD), extracellular superoxide dismutase (EC-SOD), and glutathione reductase; tissue plasminogen activator (tPA); urinary plasminogen activator (urokinase); hirudin; nitric oxide synthetase; vasoactive peptides; and angiogenic peptides.

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Full	Title	Citation	Front	Review	Classification	Date	Reference	Claims	KWIC	Draw Desc	Image
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